NMR Spectroscopy

International Edition: DOI: 10.1002/anie.201510938
German Edition: DOI: 10.1002/ange.201510938

CLIP-COSY: A Clean In-Phase Experiment for the Rapid Acquisition of COSY-type Correlations

*Martin R. M. Koos, Grit Kummerlöwe, Lukas Kaltschnee, Christina M. Thiele, and Burkhard Luy**

Abstract: *The COSY experiment is an essential homonuclear 2D NMR experiment for the assignment of resonances. Its multiplet line shape, however, is often overly complicated, potentially leads to signal intensity losses, and is responsible for long minimum overall acquisition times. Herein, we present CLIP-COSY, a COSY-type experiment yielding clean in-phase peaks. It can be recorded within a few minutes and benefits from enhanced signal intensities for most cross-peaks. In combination with non-uniform sampling, the experiment times can be further reduced, and the in-phase multiplets enable the application of modern homonuclear decoupling techniques in both dimensions. As antiphase cancelations are avoided, CLIP-COSY can also be applied to macromolecules and other samples with broadened lines.*

The COSY experiment was the first 2D NMR experiment reported;[1] however, it is still invaluable for the structure elucidation of small molecules as it enables the assignment of resonances of adjacent protons in a covalently bonded network. The COSY experiment can be recorded either in a phase-insensitive or a phase-sensitive way, the latter offering higher resolution. To date, many routine applications use phase-insensitive COSY experiments to reduce the experiment duration. Because of the enhanced resolution of pure phase spectra, it would be highly desirable to be able to acquire phase-sensitive COSY spectra within a few minutes for routine NMR spectroscopy of small molecules and metabolomics-type applications. Herein, we introduce the CLIP-COSY experiment, which provides COSY data in short acquisition times without introducing unfavorable dispersion-mode signals and is compatible with homonuclear decoupling during acquisition, which can simplify the spectra.

Variants of COSY with improved phase behavior,[2] suitable signal shape for coupling measurements,[3] constant-time acquisition,[4] and relayed transfer steps have already been reported.[5] All of these experiments, including the widely used double quantum filtered COSY (DQF-COSY),[2a] acquire so-called antiphase multiplets with severe disadvantages as will be discussed in the following.

A sine-modulated antiphase FID starts at zero intensity and therefore requires a minimum acquisition of data points to avoid a severe reduction in signal intensity (Figure 1 A). Thus the digital resolution must be high enough to resolve the active coupling to avoid cancelation of the positive and negative multiplet components (Figure 1 B). Assuming typical ^{1}H–^{1}H coupling constants of $J = 4$–10 Hz, a minimum of 1024 real points typically have to be acquired for t_1 on a 600 MHz spectrometer, leading to acquisition times of at least 30 minutes per sample, independent of the signal intensity.

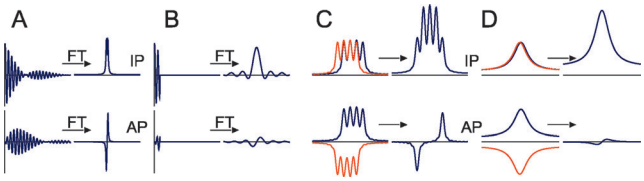

Figure 1. Comparison of in-phase (IP) and antiphase (AP) multiplet patterns. A) Cosine- and sine-modulated FIDs lead to in-phase and antiphase multiplet patterns, respectively. B) Truncated FIDs limit the signal intensities in the antiphase case. C) Overlapping multiplet components originating from passive couplings (here: doublet of doublets) partially cancel antiphase signals. D) Linewidths that are broader than the underlying coupling attenuate antiphase signals.

A second drawback emerges in the case of unresolved multiplet components: Passive couplings lead to the partial cancelation of positive and negative multiplet components, reducing the sensitivity (Figure 1 C). Similarly, broad lines originating from field inhomogeneities, exchange broadening, or fast transverse relaxation lead to cancelation of antiphase signals (Figure 1 D). Therefore, for fast acquisition of spectra and to avoid cancelation artefacts, a COSY-type pulse sequence with in-phase multiplets is required.

Several in-phase variants of COSY have already been reported: SUPER-COSY[6] provides absorptive in-phase cross-peaks but leads to undesired dispersive antiphase

[*] M. R. M. Koos, Prof. Dr. B. Luy
Institut für Organische Chemie and Institut für Biologische Grenzflächen 4—Magnetische Resonanz
Karlsruher Institut für Technologie (KIT)
Fritz-Haber-Weg 6, 76131 Karlsruhe (Germany)
E-mail: Burkhard.Luy@kit.edu
Homepage: http://ioc.kit.edu/luy
Dr. G. Kummerlöwe
Institut für Organische Chemie
Technische Universität München
Lichtenbergstrasse 4, 85747 Garching (Germany)
L. Kaltschnee, Prof. Dr. C. M. Thiele
Clemens-Schöpf-Institut für Organische Chemie und Biochemie
Technische Universität Darmstadt
Alarich-Weiss-Strasse 16, 64287 Darmstadt (Germany)

Supporting information (sample composition, experiment parameters, and pulse program source code) and the ORCID identification numbers for the authors of this article can be found under http://dx.doi.org/10.1002/anie.201510938.

diagonal peaks. ISECR-COSY[7] results in a similar behavior in the directly detected dimension. DQF-ISECR-COSY[7] finally provides the desired absorptive cross- and diagonal peaks, but requires a large number of scans per increment to be acquired. IP-COSY[8] also leads to the desired signal shapes, but the constant-time approach in the indirect dimension limits the accessible resolution and potentially distorts the peak intensities.

As none of the published in-phase experiments enables the rapid acquisition of COSY-type correlations, we designed a pulse sequence based on the general scheme introduced by Thrippleton and Keeler.[9,12] In the presented clean in-phase COSY (CLIP-COSY) experiment (Figure 2A), in-phase to in-phase coherence transfer between directly coupled spins is ensured using a perfect echo sequence[13] as the mixing element. Antiphase and zero quantum contributions are removed by two single-scan z-filter elements flanking this mixing period.

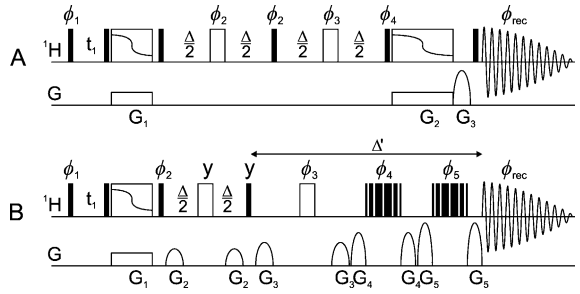

Figure 2. A) CLIP-COSY pulse sequence. The experiment can be acquired with a single scan per F_1 point; for additional scans, the pulse phases were $\phi_1=(x)_4(-x)_4$; $\phi_2=y, -y, -y, y$; $\phi_3=-y, -x, -y, -x$; $\phi_4=x, x, -x, -x$; $\phi_{rec}=x, -x, -x, x, -x, x, x, -x$. The States-TPPI progression was performed on ϕ_1. Pulse phases are x unless denoted otherwise. Filled rectangles represent 90° pulses, and open rectangles represent 180° pulses; open rectangles with frequency sweep and simultaneous application of gradients indicate z-filters.[9] The version with shaped pulses can be found in the Supporting Information. B) CLIP-COSY pulse sequence modified for solvent suppression and optimized for large molecules, such as proteins in H_2O. The second z-filter element has been replaced by excitation sculpting[10] using a binomial 3-9-19 sequence;[11] pulse phases have been adapted to avoid $-z$ magnetization; $\phi_1=x$; $\phi_2=x, -x$; $\phi_3=y, -y$; $\phi_4=(x)_4(y)_4(-x)_4(-y)_4$; $\phi_5=(x)_2(y)_2(-x)_2(-y)_2$; $\phi_{rec}=x, -x$ with States-TPPI progression of ϕ_1.

The previously published IP-COSY[8] method can be seen as a nested constant-time variant of CLIP-COSY, optimized for systems where resolution is limited by the inherent linewidth. The CLIP-COSY pulse sequence can be applied using a single scan per increment and has full flexibility concerning the acquisition parameters in both dimensions. Water suppression may be applied either by presaturation during the recovery delay or by the addition of WATERGATE,[11,14] excitation sculpting,[10] or PE WATERGATE[15] before acquisition.

The performance of the Thrippleton–Keeler z-filters increases with their duration, but polarization transfer by nuclear Overhauser enhancement (NOE), chemical exchange, or conformational interconversion and TOCSY-type transfer between strongly coupled spins may occur during this period, leading to artifacts (see the Supporting Information). Therefore, a compromise between short duration and good performance has to be found. The desired adiabatically swept inversion can be achieved by CHIRP[16] or BIP/BIBOP-type pulses[17] or by specific, optimal control (OC) derived pulses. Simulations have shown that quasi-adiabatic pulses are highly efficient down to a length of about 5 ms. For shorter pulses, OC pulses have slightly better artifact suppression efficiencies (unpublished results). In our experience, the best results for CLIP-COSY are obtained with z-filters of 0.25–0.5 ms for large molecules such as proteins and with filters of 10–20 ms for small molecules with slow NOE build-up.

In Figure 3, a conventional DQF-COSY, a variant of the IP-COSY with the Thrippleton–Keeler z-filter, and a TOCSY experiment with a short mixing time are compared with CLIP-COSY (see also the Supporting Information, Figures S4–S9).

The DQF-COSY spectrum, although recorded with high resolution in the indirect dimension, has the lowest intensity. The spectrum had to be scaled by a factor of four to show the main cross-peaks, and some of the cross-peaks are still missing compared to the in-phase spectra. The IP-COSY, acquired with an overall mixing period of $2(T_c+T_m)=52$ ms as a good compromise between efficient coherence transfer and constant-time resolution, achieves significantly better cross-peak intensities. The CLIP-COSY spectra are of even higher intensity owing to the independently adjustable mixing period. Finally, the DIPSI-2 TOCSY experiment enables the acquisition of intense spectra with a short mixing time of 35 ms, but a number of cross-peaks from relayed correlations are visible, which are undesired in COSY-type applications.

The comparison also shows the dramatic reduction of the overall experiment time that can be achieved without a significant compromise in F_1 resolution. CLIP-COSY can be easily combined with non-uniform sampling (NUS) methods,[18] such as compressed sensing. In this case, it is sufficient to acquire 76 data points for t_1, corresponding to 256 real data points with a NUS density of 30%, further extended to 512 points by linear prediction, for collecting a highly resolved and well-interpretable CLIP-COSY spectrum in three minutes (Figure 3D). It should further be noted that the CLIP-COSY experiment enables a drastic reduction of the recovery delay (see, e.g., the spectrum of rebaudiosoide A in Figure S10A). To facilitate the comparison of signal intensities, this was avoided in Figure 3.

As CLIP-COSY spectra feature full in-phase multiplets, homonuclear decoupling methods for pure shift spectra[19] can directly be applied in both dimensions. As a proof of principle, we recorded a CLIP-COSY spectrum with F_2-PSYCHE decoupling[19b] for menthol (Figure S12).

The CLIP-COSY method can be conveniently applied to molecules dissolved in water using the mentioned water suppression schemes. For protein samples, the second z-filter can be replaced by an excitation sculpting element as for large molecules, zero quantum artifacts are less of an issue (Figure 2B). In Figure 4, the fingerprint region of hen egg-white

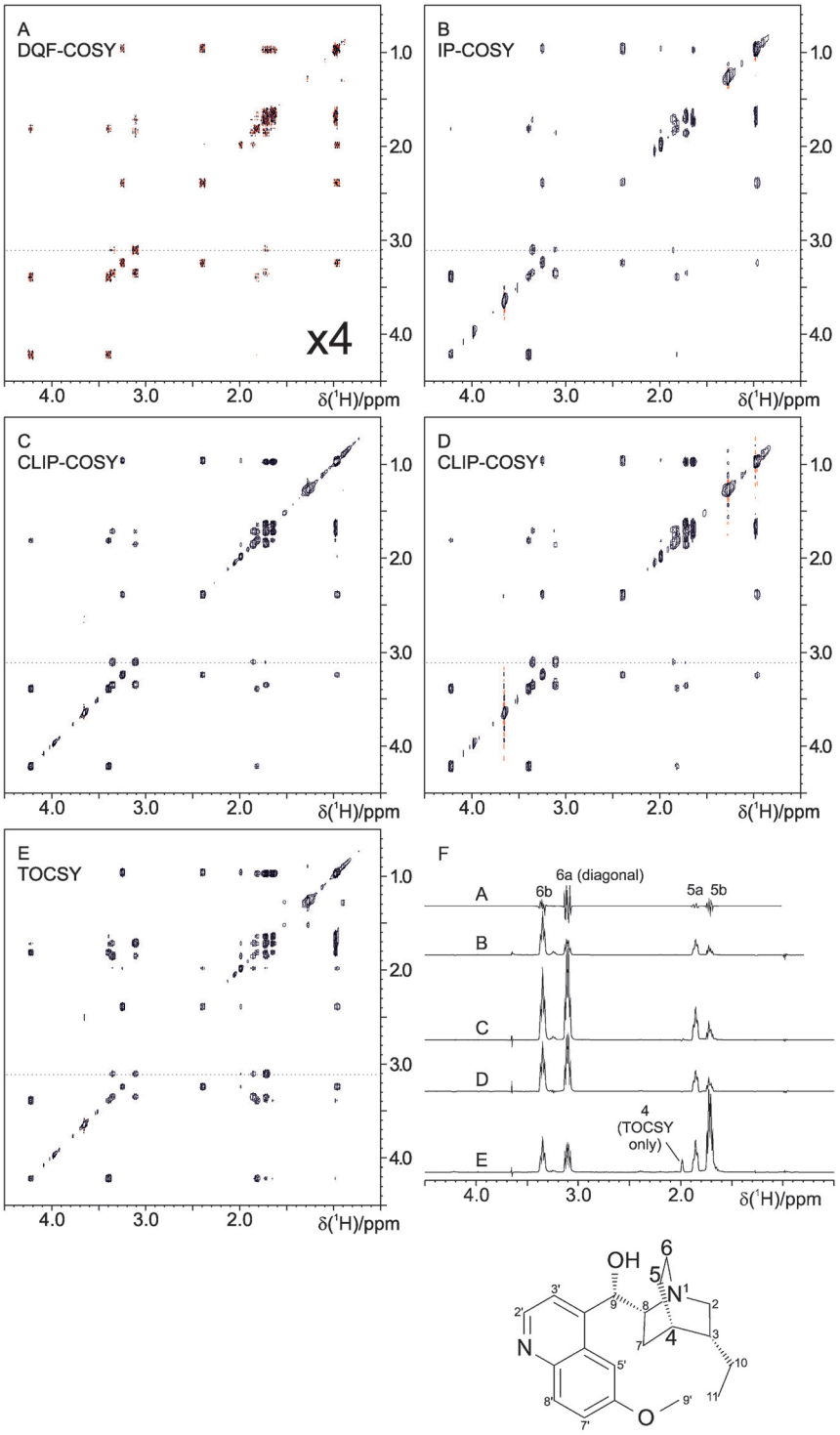

Figure 3. Aliphatic regions of example spectra acquired for 13.7 mM hydroquinidine in $CDCl_3$. A) Conventional DQF-COSY, 4096×1024 points recorded in 32 min 46 s. The peak intensities were multiplied by a factor of four to show the main cross-peaks. B) IP-COSY with the *z*-filter, 4096×384 points recorded in 13 min 3 s. The F_1 resolution is limited by the IP-COSY's constant time/overall mixing period of 52 ms. C) CLIP-COSY, 4096×1024 points recorded in 34 min 18 s using an overall mixing period of $2\Delta = 33.33$ ms. D) CLIP-COSY, 4096×76 non-uniformly sampled points (30% NUS), recorded in 2 min 59 s, reconstructed to 4096×512 points using compressed sensing and linear prediction; $2\Delta = 33.33$ ms. E) TOCSY with the *z*-filter, 4096×1024 points recorded in 34 min 24 s using DIPSI-2 with a mixing time of 34.5 ms. F) F_2 traces extracted from spectra (A)–(E) at 3.11 ppm as indicated by the dashed gray line. Spectra A, C, and E were acquired at the same resolution. For the signal assignment, see the Supporting Information.

lysozyme, which consists of 129 amino acids, is shown as an example.

The in-phase COSY variants show their full strength when lines are broadened by exchange or fast transverse relaxation. In such cases, DQF-COSY experiments suffer from prohibitive signal intensity losses. As demonstrated for *N*-methyl-4-piperidinol, which shows broadened lines owing to ring inversion,[20] the sensitivity advantage obtained with the in-phase COSY techniques can be quite drastic (see Figure 5; for the full spectra, see Figure S11).

For a critical evaluation of the CLIP-COSY method, the two potential drawbacks also have to be discussed in detail: Because of the central in-phase to in-phase coherence transfer step, in contrast to conventional COSY experiments, it is necessary to specify a mixing period of a certain duration. This implies that the coherence transfer will vary between different mixing delays Δ and will depend on the actual spin system. In the weak-coupling limit and neglecting relaxation, integrated cross-peak intensities in the CLIP-COSY are described by Eq. (1), with the active coupling J_{12} and

$$I_{12} = \sin^2\pi J_{12}\Delta \prod_{i\neq 1,2}^{n} \cos\pi J_{1i}\Delta \prod_{j\neq 1,2}^{m} \cos\pi J_{2j}\Delta \qquad (1)$$

the passive couplings J_{1i} and J_{2j} to the $(n-2)$ and $(m-2)$ neighboring spins of corresponding spin systems.

In theory, for a two-spin system, mixing times with Δ up to 50 ms should be practical. However, in our studies, we found that delays of $\Delta = 15$–25 ms are a better compromise for more complex coupling networks. In most applications with active coupling constants of $J > 3$ Hz, mixing times of around $\Delta = 15$ ms are advantageous whereas the detection of weaker couplings might require longer mixing times. In contrast to CT experiments such as IP-COSY, such short mixing periods do not limit F_1 resolution.

Aside from variations in the signal intensities, the specified mixing period also potentially leads to relayed cross-peaks if at least two spins of the spin system are strongly coupled. This effect has been discussed previously and is common to all in-phase COSY approaches (see Ref. [8] and the Supporting Information). Finally, it should

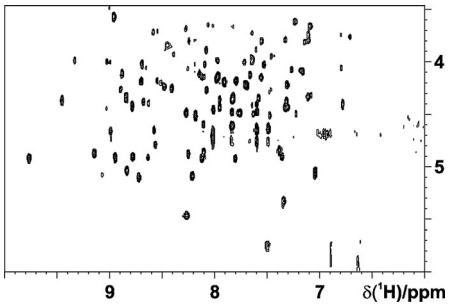

Figure 4. H^N–H^α fingerprint region of a CLIP-COSY spectrum of 1 mM lysozyme in 90% H_2O/10% D_2O using the pulse sequence shown in Figure 2B. The transfer delays were set to $\Delta = 16.7$ ms and $\Delta' = 25$ ms; 4096×512 points were recorded in 1 h 27 min, with four transients per F_1 point.

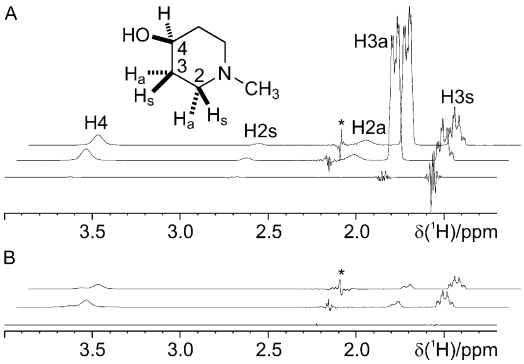

Figure 5. F_2 traces extracted from CLIP-COSY (upper traces), IP-COSY (middle traces), and DQF-COSY (lower traces) spectra, collected for *N*-methyl-4-piperidinol at 283 K. The traces were extracted at F_1 frequencies of A) H^3s and B) H^4. All traces were plotted at the same noise level. For the CLIP- and IP-COSY spectra, 64 F_1 points were collected, and the same effective mixing time was used, $2\Delta = 2(T_c + T_m) = 52$ ms. The DQF-COSY spectrum was collected with 512 F_1 points to avoid truncation (8-fold experiment duration). Signals marked with asterisks are caused by t_1 noise at the N–CH_3 frequency.

be mentioned that the performance of the *z*-filter inherently must break down close to the diagonal,[9] and corresponding multiplets might get distorted. Such distortions, however, are inherent to all COSY-type experiments for strongly coupled spins.

In summary, the CLIP-COSY method has been introduced as an easy-to-handle COSY experiment that provides high quality in-phase multiplet patterns. The main advantage of the CLIP-COSY experiment is the possibility to rapidly acquire spectra with full absorptive line shapes in a few minutes, especially when combined with non-uniform sampling methods. For signals with line broadening due to exchange, large improvements in sensitivity can be achieved compared to DQF-COSY experiments. The sequence does not limit the F_1 resolution and enables the combination of COSY with modern homodecoupled acquisition methods as for TOCSY/NOESY experiments.[19a,21] Owing to the strongly reduced experiment time, the acquisition of absorptive COSY-type correlation spectra will be amenable as a routine NMR method and might also find applications in metabolomics studies and quality control as a sensitive high-resolution 2D experiment.

Acknowledgements

B.L. and M.R.M.K. thank the Deutsche Forschungsgemeinschaft (LU 835/6-2, LU 835/13-1, instrumentation facility Pro²NMR), the HGF program BIFTM and the Fonds der Chemischen Industrie for financial support. Funding by the ERC (257041) and by the Merck Foundation for Arts and Science is acknowledged by C.M.T. and L.K.

Keywords: analytical methods · COSY · NMR spectroscopy · perfect echo · rapid acquisition

How to cite: *Angew. Chem. Int. Ed.* **2016**, *55*, 7655–7659
Angew. Chem. **2016**, *128*, 7785–7789

[1] W. P. Aue, E. Bartholdi, R. R. Ernst, *J. Chem. Phys.* **1976**, *64*, 2229–2246.
[2] a) U. Piantini, O. W. Sørensen, R. R. Ernst, *J. Am. Chem. Soc.* **1982**, *104*, 6800–6801; b) D. Marion, A. Bax, *J. Magn. Reson.* **1988**, *80*, 528–533; c) M. Rance, O. W. Sørensen, G. Bodenhausen, G. Wagner, R. R. Ernst, K. Wüthrich, *Biochem. Biophys. Res. Commun.* **1983**, *117*, 479–485; d) H. Oschkinat, A. Pastore, P. Pfändler, G. Bodenhausen, *J. Magn. Reson.* **1986**, *69*, 559–566; e) A. J. Pell, R. A. E. Edden, J. Keeler, *Magn. Reson. Chem.* **2007**, *45*, 296–316.
[3] a) C. Griesinger, O. W. Sørensen, R. R. Ernst, *J. Magn. Reson.* **1987**, *75*, 474–492; b) C. Griesinger, O. W. Sørensen, R. R. Ernst, *J. Chem. Phys.* **1986**, *85*, 6837–6852; c) C. Griesinger, O. W. Sørensen, R. R. Ernst, *J. Am. Chem. Soc.* **1985**, *107*, 6394–6396; d) L. Mueller, *J. Magn. Reson.* **1987**, *72*, 191–196; e) A. Bax, R. Freeman, *J. Magn. Reson.* **1981**, *44*, 542–561.
[4] a) S. Berger, *Spectrosc. Lett.* **2000**, *33*, 1–8; b) M. E. Girvin, *J. Magn. Reson. Ser. A* **1994**, *108*, 99–102; c) M. Rance, G. Wagner, O. W. Sørensen, K. Wüthrich, R. R. Ernst, *J. Magn. Reson.* **1984**, *59*, 250–261; d) J. A. Aguilar, A. A. Colbourne, J. Cassani, M. Nilsson, G. A. Morris, *Angew. Chem. Int. Ed.* **2012**, *51*, 6460–6463; *Angew. Chem.* **2012**, *124*, 6566–6569.
[5] G. Eich, G. Bodenhausen, R. R. Ernst, *J. Am. Chem. Soc.* **1982**, *104*, 3731–3732.
[6] A. Kumar, R. V. Hosur, K. Chandrasekhar, *J. Magn. Reson.* **1984**, *60*, 143–148.
[7] S. Talluri, H. A. Scheraga, *J. Magn. Reson.* **1990**, *86*, 1–10.
[8] Y. Xia, G. Legge, K.-Y. Jun, Y. Qi, H. Lee, X. Gao, *Magn. Reson. Chem.* **2005**, *43*, 372–379.
[9] M. J. Thrippleton, J. Keeler, *Angew. Chem. Int. Ed.* **2003**, *42*, 3938–3941; *Angew. Chem.* **2003**, *115*, 4068–4071.
[10] T.-L. Hwang, A. J. Shaka, *J. Magn. Reson. Ser. A* **1995**, *112*, 275–279.
[11] M. Liu, X. Mao, C. Ye, H. Huang, J. K. Nicholson, J. C. Lindon, *J. Magn. Reson.* **1998**, *132*, 125–129.
[12] K. E. Cano, M. J. Thrippleton, J. Keeler, A. J. Shaka, *J. Magn. Reson.* **2004**, *167*, 291–297.
[13] a) L. Kaltschnee, A. Kolmer, I. Timári, V. Schmidts, R. W. Adams, M. Nilsson, K. E. Kövér, G. A. Morris, C. M. Thiele, *Chem. Commun.* **2014**, *50*, 15702–15705; b) A. M. Torres, G. Zheng, W. S. Price, *Magn. Reson. Chem.* **2010**, *48*, 129–133; c) P. C. M. van Zijl, C. T. W. Moonen, M. von Kienlin, *J. Magn. Reson.* **1990**, *89*, 28–40; d) K. Takegoshi, K. Ogura, K. Hikichi, *J. Magn. Reson.* **1989**, *84*, 611–615.
[14] M. Piotto, V. Saudek, V. Sklenář, *J. Biomol. NMR* **1992**, *2*, 661–665.

[15] R. W. Adams, C. M. Holroyd, J. A. Aguilar, M. Nilsson, G. A. Morris, *Chem. Commun.* **2013**, *49*, 358 – 360.
[16] a) J.-M. Böhlen, I. Burghardt, M. Rey, G. Bodenhausen, *J. Magn. Reson.* **1990**, *90*, 183 – 191; b) J.-M. Böhlen, M. Rey, G. Bodenhausen, *J. Magn. Reson.* **1989**, *84*, 191 – 197.
[17] a) K. Kobzar, T. E. Skinner, N. Khaneja, S. J. Glaser, B. Luy, *J. Magn. Reson.* **2008**, *194*, 58 – 66; b) K. Kobzar, T. E. Skinner, N. Khaneja, S. Glaser, B. Luy, *J. Magn. Reson.* **2004**, *170*, 236 – 243; c) M. A. Smith, H. Hu, A. J. Shaka, *J. Magn. Reson.* **2001**, *151*, 269 – 283.
[18] M. Mobli, J. C. Hoch, *Prog. Nucl. Magn. Reson. Spectrosc.* **2014**, *83*, 21 – 41.
[19] a) N. H. Meyer, K. Zangger, *Angew. Chem. Int. Ed.* **2013**, *52*, 7143 – 7146; *Angew. Chem.* **2013**, *125*, 7283 – 7286; b) M. Foroozandeh, R. W. Adams, N. J. Meharry, D. Jeannerat, M. Nilsson, G. A. Morris, *Angew. Chem. Int. Ed.* **2014**, *53*, 6990 – 6992; *Angew. Chem.* **2014**, *126*, 7110 – 7112; c) K. Zangger, H. Sterk, *J. Magn. Reson.* **1997**, *124*, 486 – 489; d) J. R. Garbow, D. P. Weitekamp, A. Pines, *Chem. Phys. Lett.* **1982**, *93*, 504 – 509; e) T. Reinsperger, B. Luy, *J. Magn. Reson.* **2014**, *239*, 110 – 120; f) I. Timári, L. Kaltschnee, A. Kolmer, R. W. Adams, M. Nilsson, C. M. Thiele, G. A. Morris, K. E. Kövér, *J. Magn. Reson.* **2014**, *239*, 130 – 138.
[20] a) C.-Y. Chen, R. J. W. Le Fèvre, *Tetrahedron Lett.* **1965**, *6*, 4057 – 4063; b) T. A. Crabb, A. R. Katrizky, *Adv. Heterocycl. Chem.* **1984**, *36*, 1 – 173.
[21] a) G. A. Morris, J. A. Aguilar, R. Evans, S. Haiber, M. Nilsson, *J. Am. Chem. Soc.* **2010**, *132*, 12770 – 12772; b) J. J. Koivisto, *Chem. Commun.* **2013**, *49*, 96 – 98; c) M. Foroozandeh, R. W. Adams, M. Nilsson, G. A. Morris, *J. Am. Chem. Soc.* **2014**, *136*, 11867 – 11869; d) L. Castañar, P. Nolis, A. Virgili, T. Parella, *Chem. Eur. J.* **2013**, *19*, 17283 – 17286.

Received: November 25, 2015
Revised: February 5, 2016
Published online: May 19, 2016

